Determination of MIC, MPC, and MSW of *Ilex paraguariensis* Against Non-Typhoidal *Salmonella* with Identification of the Mechanisms of Resistance and Pathogenicity Factors

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Research note

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Abstract

Objective: This study investigated the antibacterial activity of *Ilex paraguariensis* extracts against 32 different strains of non-typhoidal *Salmonella* (NTS) through the determination of the Minimum Inhibitory Concentration (MIC), Mutant Prevention Concentration (MPC), Mutant Selection Window (MSW), and the detection of virulence genes by multiplex PCR assays.

Results: The MIC values of *Ilex paraguariensis* against *Salmonella* spp. strains varied between 0.78 mg/ml and 6.25 mg/ml with a MIC₉₀ of 3.12 mg/ml. The highest MPC in this study was 48 mg/ml yielding a Mutant Selection Window of 41.75 mg/ml. The MSW values of the remaining strains varied between 1.56 and 8.87 mg/ml. Genes of pathogenicity detected in *Salmonella* spp. isolates were most commonly the *stn*, *sdiA*, *invA*, *sopB*, *invH*, and *sopE* genes. The antibacterial activity of Yerba Mate extracts was not affected by the antimicrobial resistance patterns or pathogenicity genes expressed. More work is needed to identify the active antibacterial compound(s) responsible for the antibacterial activity.

Introduction

Salmonella typhi- and *Salmonella paratyphi*-associated enteric fevers cause severe diseases, affecting mainly underdeveloped areas, while other non-typhoidal *Salmonella* (NTS) infections are more self-limiting but with a wide-reaching prevalence (Eng et al., 2015). Enteric fevers are major causes of morbidity and mortality (Sánchez-Vargas et al., 2011). Non-typhoidal *Salmonella* remains an important cause of morbidity with *Salmonella enteritidis* and *Salmonella typhimurium* being the most prevalent serotypes (Eng et al., 2015). NTS causes an estimated 93.8 million cases of acute gastroenteritis worldwide each year (Balasubramanian et al., 2019; Al-Rifai et al., 2020). NTS can be transmitted via food contaminated by animal feces (Crump et al., 2015). The severity of salmonellosis depends on the host and on the associated virulence factors that are controlled by chromosomal or plasmidic genes. Virulence genes can be found clustered together on 'pathogenicity islands' which are activated at a specific time during the course of infection. Thus, a single pathogenicity island can make a specific serotype pathogenic, such as the role of *Salmonella* pathogenicity island 1 (SPI-1) in the penetration of intestinal epithelial cells, the role of SPI 2, 3 and 4 in bacterial survival, and the role of SPI 5 in inflammation (Marcus et al., 2000).

In contrast to enteric fevers, gastroenteritis caused by NTS is usually a self-limiting infection that does not require antibiotic treatment. Furthermore, persistent digestive symptoms are more common among patients treated with antibiotics (9.5%) as opposed to those not treated with antibiotics (2.9%) (Giorgio, 2000). However, antibiotics become necessary in case of complications, such as meningitis and septicemia (Medalla et al., 2017). Unfortunately, decades of use of these antibiotics resulted in the emergence of multi-drug resistant *Salmonella*, resulting in increased mortality.

Ilex paraguariensis, known as Yerba mate (YM), is a plant originally from South America's subtropical region. YM is considered as an alternative to coffee and tea and is consumed by millions of people worldwide. There are different bioactive compounds in YM leaves associated with many health benefits including antioxidant properties, hypoglycemic effects, fat loss properties and most importantly its antimicrobial activity (Bastos et al., 2007; Fayad et al., 2020; Kungel et al., 2018; Nouredine et al., 2018).

The aim of this study was to evaluate the antimicrobial activity of YM extracts against non-typhoidal *Salmonella* isolates through the determination of the Minimum Inhibitory Concentrations (MIC), Mutant Prevention Concentration and Mutant Selection Window (MPC and MSW), and the detection of virulence genes by multiplex-PCR assay.

Materials And Methods

Bacterial strains and antibiotic susceptibility: Thirty-two clinical isolates of *Salmonella* spp. were isolated from Lebanese and Beninese patients. *Salmonella* isolates were identified using API 20E system (Biomérieux, France) after isolation on Salmonella Shigella agar (SS) (Biorad- Germany). Antibiotic Susceptibility Testing was performed using the Kirby Bauer technique with different antibiotics disks (Biorad, Germany). The results were interpreted according to EUCAST guidelines (2017). The susceptibility of the isolates to colistin was determined using the Microdilution method (CLSI, 2018)

Phenotypic detection of ESBL and AmpC

Phenotypic detection of extended spectrum beta-lactamase (ESBL) producing bacteria and AmpC beta-lactamases were performed as explained in Dandachi et al. (2018) using the double-disk synergy test or the ceftiofloxacin disk test (Black et al., 2005) respectively. For the detection of carbapenemases, the carbapenemase NP test was used as per the manufacturer instructions (Cunningham et al., 2017) to distinguish between KPC, Oxa-48, VIM, IMP, and NDM.

Molecular characterization of beta-Lactamase encoding genes

Isolates that showed ceftiofloxacin resistance or a keyhole effect were further studied using real-time PCR as detailed in Dandachi et al. (2018). Briefly, DNA was extracted from isolates using EZ1 DNA extraction kits (Qiagen, France) and real-time PCR was used for the detection of CTX-M, TEM, SHV, and other resistance genes (Roschanski et al., 2014).

Determination of the Minimum Inhibitory Concentration

The Minimum Inhibitory Concentration (MIC) was performed using broth microdilution method as described in the Clinical and Laboratory Standards Institute (CLSI, 2018). Briefly the highest concentration of the antimicrobial agent was added to the first well of a 96-well plate and a serial dilution was performed. A volume of 100 µl of a 10⁶ CFU/ml bacterial suspension was dispensed into all the wells except for the negative control. The plate was incubated overnight and MIC was recorded as the

concentration of the first well that shows no bacterial growth. MIC 90 is defined as the lowest concentration of the extract at which 90% of the bacterial isolates were inhibited.

Mutant Prevention Concentration

The Mutant Prevention Concentration (MPC) was determined using the agar plate dilution method as described by Pasquali & Manfreda (2007). Briefly, the antimicrobial agents were incorporated into Mueller Hinton Agar (MHA) plates to create a concentration gradient from 1000-0.1 µg/ml. The bacterial suspension was then concentrated to 10^{10} CFU/ml and inoculated on the entire range of antibiotic containing MHA plates. The plates were incubated and the concentration of the first plate that showed no growth after 48 hours was considered as the MPC. All the tests were performed in duplicate.

Detection of virulence genes by Polymerase Chain Reaction.

Salmonella strains were cultured overnight at 37°C in brain heart infusion broth (Oxoid, United Kingdom). An aliquot of 1 ml of each bacterial culture was separated for DNA extraction by heat treatment (Borges et al., 2013). The PCR experiments were conducted in individual reactions using primers for the following genes: *sdhA*, *invA*, *sopB*, *invH*, *sopE*, *pefA*, and *sefC* (Choudhury et al., 2016). The cycling program was performed in the CFX-96 thermal cycler (BioRad, Germany). The amplified products were separated by electrophoresis in a 1.2% agarose gel and stained with ethidium bromide. Fragments were trans-illuminated with UV light. *E. coli* ATCC 25922 (stn -ve) and *S. enteritidis* ATCC 13076 (stn +ve) were used as negative and positive controls respectively for all PCR reactions. A control containing the PCR mix without extracted DNA was also added in all PCR runs. All isolates having colistin MIC > 2mg/ml were subjected to standard PCR for the detection of *mcr-1* colistin resistant gene (Rebelo et al. (2018).

Aqueous YM Extraction

Commercial YM dried leaves (brand AMANDA) were purchased from the local market and an aqueous extraction was performed as explained in Nouredine et al. (2018). In brief, the leaves were blended and mixed with distilled water at a ratio of 3.6:1 ml/g. The mixture was heated at 70°C for 2 hours. Extracts were filtered using Whatman (1M) filter papers, dialyzed against distilled water for 48 hours, centrifuged at 3325 G, concentrated using a SpeedVacuum concentrator (Thermo Fisher Scientific, USA), and stored at 4 °C.

Results

The MIC values of *Ilex paraguariensis* against *Salmonella spp.* strains varied between 0.78 mg/ml and 6.25 mg/ml with a MIC 90 of 3.12 mg/ml (Table 1). ESBL strains had MIC values of 0.78 mg/ml and 1.56 mg/ml (Table 1). All *Salmonella* strains manifested full susceptibility to carbapenems (ertapenem, imipenem) and trimethoprim-sulfamethoxazole. However, only 84% were susceptible to 3rd generation cephalosporins (cefotaxime and ceftazidime) and 69% were susceptible to ampicillin (Table S1). The phenotypic and genotypic mechanisms of resistance to beta-lactam antibiotics as well as the presence of

mcr-1 responsible for resistance to colistin in *Salmonella* spp. isolates were studied (Table 2). Five isolates were associated with the production of at least one ESBL gene. Of these isolates, four produced 2 types of ESBL genes, one produced all ESBL genes (TEM, SHV, and CTX-M). All five isolates gave a positive ESBL phenotypic test showing synergy between clavulanic acid and at least one of the three 3rd generation cephalosporins tested. TEM and SHV genes were found in 9.4% and 12.5% of the *Salmonella* strains accounting for 40% and 80% respectively of ESBL producing strains. CTX-M was consistently present in all ESBL producing isolates (isolates 41, 51, 61, 62, 65). All *Salmonella* isolates tested were susceptible to colistin antibiotic with MIC 90 of 0.25 mg/l and mcr-1 gene was not detected (Table 2).

Table 1

Minimum Inhibitory Concentrations of *Ilex paraguariensis* against different strains of non-typhoidal *Salmonella*. Mutant Prevention Concentration (MPC) and Mutant Selection Window (MSW) of YM were studied on selected *Salmonella* spp. isolates (strain numbers 11, 20, 34, 35, 51, 56, 59, 60, 65).

<i>Salmonella</i> Strain Number	MIC (mg/ml)	MPC (mg/ml)	MSW (mg/ml)
1	3.12		
4	1.56		
11	3.12	12	8.87
12	3.12		
14	1.56		
17	3.12		
20	1.56	6.25	4.69
22	3.12		
26	3.12		
29	1.56		
32	1.56		
33	0.78		
34	1.56	3.25	1.69
35	1.56	6.25	4.69
36	0.78		
38	3.12		
41*	0.78		
45	1.56		
49	3.12		
50	1.56		
51*	0.78	3.12	2.34
52	1.56		
54	1.56		

* ESBL producing strain; **MIC 90 is the concentration at which 90% of the strains MIC lie.

<i>Salmonella</i> Strain Number	MIC (mg/ml)	MPC (mg/ml)	MSW (mg/ml)
55	0.78		
56	1.56	3.12	1.56
59	1.56	3.12	1.56
60	6.25	48	41.75
61*	0.78		
62*	0.78		
64	1.56		
65*	1.56	3.12	1.56
66	3.12		
MIC 90**	3.12		
* ESBL producing strain; **MIC 90 is the concentration at which 90% of the strains MIC lie.			

Table 2
Phenotypic and genotypic mechanisms of resistance to beta-lactam antibiotics and colistin in
Salmonella spp. isolates.

<i>Salmonella</i> Strain	Resistance to Beta-Lactams				Resistance to Colistin	
	Genes of resistance detected by PCR			Phenotypic resistance	Gene of resistance	Phenotypic resistance
	TEM	SHV	CTX-M	ESBL	mcr-1	MIC mg/l
1	-	-	-	-	-	0.12 (S)
4	-	-	-	-	-	0.25 (S)
11	-	-	-	-	-	0.5 (S)
12	-	-	-	-	-	0.06 (S)
14	-	-	-	-	-	0.12 (S)
17	-	-	-	-	-	0.06 (S)
20	-	-	-	-	-	0.06 (S)
22	-	-	-	-	-	0.06 (S)
26	+	-	-	-	-	0.25 (S)
29	-	-	-	-	-	0.06 (S)
32	-	-	-	-	-	0.06 (S)
33	-	-	-	-	-	0.25 (S)
34	-	-	-	-	-	0.12v (S)
35	-	-	-	-	-	0.12 (S)
36	-	-	-	-	-	0.03 (S)
38	-	-	-	-	-	0.5 (S)
41	-	+	+	+	-	0.03 (S)
45	-	-	-	-	-	0.12 (S)
49	-	-	-	-	-	0.06 (S)
50	-	-	-	-	-	0.06 (S)
51	+	+	+	+	-	0.06 (S)
52	-	-	-	-	-	0.12 (S)

S: susceptible; MIC 90 is the concentration at which 90% of the strains MIC lie.

Resistance to Beta-Lactams					Resistance to Colistin	
54	-	-	-	-	-	0.06 (S)
55	-	-	-	-	-	0.12 (S)
56	-	-	-	-	-	0.12 (S)
59	-	-	-	-	-	0.06 (S)
60	-	-	-	-	-	0.03 (S)
61	+	-	+	+	-	0.25 (S)
62	-	+	+	+	-	0.25 (S)
64	-	-	-	-	-	0.03 (S)
65	-	+	+	+	-	0.12 (S)
66	-	-	-	-	-	0.03 (S)
Positivity	9.4%	12.5%	15.6%	15.6%	0%	MIC 90 = 0.25
S: susceptible; MIC 90 is the concentration at which 90% of the strains MIC lie.						

The Mutant Prevention Concentrations of *Ilex paraguariensis* against non-typhoidal *Salmonella* isolates were studied on selected *Salmonella* spp. isolates (Table 1). The highest MPC found in this study is that of strain 60 (48 mg/ml) yielding a Mutant Selection Window of 41.75 mg/ml. The MSW values of the remaining strains varied between 1.56–8.87 mg/ml. Table 3 shows the different pathogenicity genes detected in *Salmonella* isolates. Most commonly, the genes *stn*, *sdiA*, *invA*, *sopB*, *invH*, and *sopE* were detected. Twenty-eight isolates (87.5%) were associated with the detection of more than one gene of resistance. *Stn* gene was found in all the isolates and was followed by the gene *sdiA* with 81.2% of detection rate (Table 3).

Table 3

Genes of pathogenicity detected in Salmonella isolates studied. PCR experiments were conducted using primers for stn, sdiA, invA, sopB, invH, sopE, pefA, and sefC. The amplified products were separated by electrophoresis in a 1.2% agarose gel and stained with ethidium bromide.

Serotypes		Genes							
Strain		Stn	sdiA	invA	sopB	invH	sopE	pefA	sefC
1	<i>S. enteritidis</i>	+	+	+	+	+	+	-	-
4	<i>S. enteritidis</i>	+	+	+	+	+	+	-	-
11	<i>S. enteritidis</i>	+	-	+	+	+	+	-	-
12	<i>S. enteritidis</i>	+	+	+	+	+	+	-	-
14	<i>S. enteritidis</i>	+	+	-	-	+	+	-	-
17	<i>S. enteritidis</i>	+	+	+	+	+	+	-	-
20	<i>S. enteritidis</i>	+	+	+	+	+	+	-	-
22	<i>S. enteritidis</i>	+	-	+	+	+	+	-	-
26	<i>S. enteritidis</i>	+	+	+	+	+	+	-	-
29	<i>S. enteritidis</i>	+	+	-	-	+	+	-	-
32	<i>S. typhimurium</i>	+	+	-	-	+	+	-	-
33	<i>S. enteritidis</i>	+	+	+	+	+	+	-	-
34	<i>S. enteritidis</i>	+	+	-	-	-	-	-	-
35	<i>S. enteritidis</i>	+	+	-	-	-	-	-	-
36	<i>S. enteritidis</i>	+	+	-	-	-	-	-	-
38	<i>S. corvalis</i>	+	-	-	-	-	-	-	-
41	<i>S. enteritidis</i>	+	+	-	-	-	-	-	-
45	<i>S. enteritidis</i>	+	+	-	-	-	-	-	-
49	<i>S. enteritidis</i>	+	+	-	-	-	-	-	-
50	<i>S. enteritidis</i>	+	+	-	-	-	-	-	-
51	<i>S. enteritidis</i>	+	+	-	-	-	-	-	-
52	<i>S. enteritidis</i>	+	+	-	-	-	-	-	-
54	<i>S. enteritidis</i>	+	-	-	-	-	-	-	-
55	<i>S. enteritidis</i>	+	+	-	-	-	-	-	-

	Serotypes	Genes							
56	<i>S. enteritidis</i>	+	+	-	-	-	-	-	-
59	<i>S. enteritidis</i>	+	-	-	-	-	-	-	-
60	<i>S. enteritidis</i>	+	+	-	-	-	-	-	-
61	<i>S. enteritidis</i>	+	-	-	-	-	-	-	-
62	<i>S. enteritidis</i>	+	+	-	-	-	-	-	-
64	<i>S. enteritidis</i>	+	+	-	-	-	-	-	-
65	<i>S. enteritidis</i>	+	+	-	-	-	-	-	-
66	<i>S. enteritidis</i>	+	+	-	-	-	-	-	-
% of positivity		100	81.2	28.1	28.1	37.5	37.5	0	0

Discussion

Non-typhoidal Salmonella infections are a major cause of diarrhea, they can even cause serious invasive salmonellosis (Wen et al., 2017). *Ilex paraguariensis* is popularly consumed in many countries and is rich in different bioactive compounds such as purine alkaloids, phenolic compounds, and saponins (Bastos et al., 2007). In the present work, YM extracts had an antibacterial activity against different *Salmonella* spp. with MIC between 0.78–3.12 mg/ml. Similarly, YM hexane and ethanol extracts showed antibacterial activity against *S. typhimurium* with MICs of 0.58 and 1.14 mg/ml respectively (Vieitez et al., 2018). Kungel et al. (2018) isolated a polysaccharide from YM with antibacterial activity against *S. enteritidis* and *S. typhimurium* (MIC 0.4 mg/ml).

YM ethanolic extracts (richer in phenolic content) were more effective against *S. enteritidis* than methanolic extracts with MICs 3.13 and 6.25 mg/ml respectively (Martin et al., 2013). Although studies suggest better potency of YM on gram-positive bacteria, the hexane and aqueous extracts of YM had MIC 50–200 µg/ml on different gram-negative bacteria (Penteado et al., 2016). The aqueous extract of YM (at 64 mg/ml) significantly inhibited the growth of *Salmonella enterica* (Rempe, 2016). YM had bacteriostatic and bactericidal activities against different serovars of *Salmonella* spp. with MICs 150–200 mg/ml (De Bona et al., 2010). *S. enteritidis* was the most sensitive among different bacteria studied with inhibition (value 8) (Girolometto et al., 2009).

Mutant Selection Window (MSW) corresponds to the danger zone of drug concentration in which the bacterium can undergo a mutation that enables it to become phenotypically resistant to that drug and to show clinical failure. Subsequently, the smaller the value of the MSW, the better is the prognosis of the treatment. YM manifested a relatively narrow MSW that was a multiple of 2 or 3 of the MIC (Table 1). The genotypic and phenotypic mechanisms of resistance of *Salmonella* isolates tested here did not correlate with a special resistance to commonly used antibiotics. ESBL producing strains were not correlated with

the highest resistance to YM. Furthermore, among the *Salmonella* isolates that showed more than 2 virulence genes, none showed a higher MIC as compared to the rest of the strains, suggesting therefore that no relationship between these two parameters exist.

Salmonella biofilms have proven to vastly contribute to resistance to a variety of clinically important antibiotics like ciprofloxacin and quinolones. From this point, there is an interest to explore plant extracts as anti-biofilm tools to reduce the risk of disease with pathogenic microorganisms (Sakarikou et al., 2020). For example, the hydrosol of *Thymbra capitata* proved to be effective against *Salmonella* biofilm (Sakarikou et al., 2020). Burdock leaves has an anti-biofilm activity against *S. typhimurium* (Liu et al., 2017). More studies are needed to further examine the interaction that YM extracts might have on the different virulence factors (such as the biofilm) of different *Salmonella* species.

Conclusion

The discovery of antimicrobial activities of plant extracts can be considered an important step towards the development of new antimicrobial agents. While the MIC of YM does not compare to antibiotics used in the medical practice, these extracts might play a crucial role in decreasing the bacterial inoculum attenuating therefore the possibility of the infection. More work is needed to identify the antibacterial compound(s) found in YM.

Limitation

A limitation of this study is the sample size of *Salmonella* strains studied with different profiles of resistance. Another is the identification of bioactive compound(s) in *Ilex paraguariensis*.

List Of Abbreviations

Invasive Non-Typhoidal Salmonella (iNTS), Minimum Inhibitory Concentration (MIC), Mueller Hinton Agar (MHA), Mutant Prevention Concentration (MPC), Mutant Selection Window (MSW), Non-Typhoidal Salmonella (NTS), Salmonella Pathogenicity Island (SPI), Yerba mate (YM).

Declarations

Ethics approval and consent to participate

All bacterial isolates were kindly provided by the CHN hospital ([Centre Hospitalier du Nord](#)), North Lebanon. The plant samples (YM) were purchased from the local market.

Consent for publication

Not applicable.

Availability of data and materials

All the remaining data generated during this study are included in the manuscript or as Supplementary Data.

Competing of Interest

The authors declare that they have no competing interests.

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Authors' contributions

KEK, RAH, AS, AK, and EF conducted experimental procedures, data analysis and manuscript preparation. VD helped in collecting samples and in manuscript revision. RAM and ZD conceived the design of the work and were involved in the analysis and interpretation of results and provided substantial revision of the manuscript. All authors read and approved the final manuscript.

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